Determining the effect of a synbiotic supplement on the microbial profile of intestinal health in turkey poults

Presented in Partial Fulfillment of the
Requirements for Graduation with Research Distinction

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2019

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ABSTRACT:

An *Eimeria* challenge may lead to coccidiosis causing morbidity and mortality, with a subclinical infection of coccidiosis causing reduced feed conversion, and reduced production. *Eimeria* is estimated to cost the United States $3 billion in total damages. Despite the severity of consequences and damages *Eimeria* causes, there are currently no great options for control and prevention. The object of this trial is to determine the effect of a synbiotic feed supplement on the performance, microbial profile, and parameters of intestinal health of turkey poults that will be subjected to mixed *Eimeria* challenge. On day of hatch (dOH), the treatments challenged with *Salmonella enterica* Enteritidis ($4x10^4$ CFU/poult) were challenged, non-nonmedicated (CNM), challenged, synbiotic supplemented (CSS), challenged, and Clinacox (1 PPM) medicated (CCM). On d16, the CNM, CSS, and CCM treatment groups were challenged with a mix of *Eimeria adenoeides* and *Eimeria meleagrimitis*. The treatments groups consisting of non-challenged, non-medicated (NCNM), non-challenged, synbiotic supplemented (NCSS), and Non-challenged, Clinacox (1 PPM) medicated (NCCM) did not receive doses of either challenge. On d21 and d28, five poults from pen (n=30 poults per group) were euthanized and lesion scored for *Eimeria.* Gastrointestinal tract (GIT) samples were collected at d21 and d28 for qPCR analysis. qPCR data was analyzed via Oneway Analysis of Variance (ANOVA) using the JMP Pro13 Software (JMP Software, SAS Inc., 2018) and significant differences between groups were compared via Student's *t*-test ($p \leq 0.05$). No samples from either d21 or d28 had a detectable level of *Salmonella* present. On d21, there was a higher fold change of the Firmicutes population in all treatment groups compared to NCNM. On d28, the synbiotic supplemental treatment groups had the highest increases for Firmicutes, while CSS had the largest increase with significant difference ($p \leq 0.05$) between CSS and CNM. On d21, among all groups relative to the NCNM there were reductions in the *Lactobacillales* (LAB) order. With the Lactobacillus genus on d21 and d28, there was a reduction among all groups relative to NCNM. Reductions among all groups occurred for the *Bifidobacterium* genus relative to NCNM. On d28, the reductions became fold increases among all groups except for CNM. For the d21 and d28 APEC analysis, there were significant differences between groups observed. These results suggest that the synbiotic supplement altered the microbial profile in turkey poults.

INTRODUCTION:

Poultry is the most consumed meat source in the world, with consumption of turkey being 17% of the poultry consumed (Waite et al., 2017). It is widely known that the intestinal health of poultry plays an important role in the overall health and wellbeing of the bird. Therefore, it is important to understand the stresses and disease that commercial poultry are susceptible to. The turkey microbiome is largely understudied (Waite et al., 2017). By understanding the turkey microbiome and the role they microbiome has in maintaining the homeostasis in poultry, strides will be made in bettering turkey health and welfare, while also increasing production and efficiency. Additionally, antibiotic usage is no longer acceptable. It is essential to find other solutions such as probiotics. This is important for a consumer as it will create a healthier product for the consumer, while maintaining consumer values.

Turkeys are exposed to pathogens, such as *Eimeria* and *Salmonella*, in commercial settings. *Eimeria* infection predisposes birds to coccidiosis, causing high inflammation in the GIT and poor performance (Shivaramaiah et al., 2011). *Salmonella* is also a concern in poultry for two main reasons. *Salmonella* will reduce the performance of birds, while increasing the likelihood of necrotic enteritis. From a food-safety standpoint, *Salmonella* is a danger to public

health (Shivaramaiah et al., 2011). In this study, the inoculation of *Salmonella* will decrease the immune response of the turkey poults and exacerbate the effects of inoculating the birds with *Eimeria.* This allows the researchers to understand the impact of diseases on the microbiome of turkeys, while examining the effects of the synbiotic supplement and an antibiotic.

Synbiotics are mixtures of prebiotics and probiotics, composed of beneficial probiotic microorganisms which benefit from the addition of prebiotics. Synbiotics work synergistically, and are notable for their benefits to an organism by improving the survival and boosting colonies of live microbials in the GIT, and activating the metabolism of health-promoting bacteria, while overall improving the welfare of the organism (Markowiak and Śliżewska, 2018). By bolstering the intestinal health of the turkey poults through synbiotics, it is expected to see healthier birds and a beneficial microbial community in the GIT.

MATERIALS AND METHODS:

Six treatment groups consisted of non-challenged, non-medicated (NCNM), non-challenged, synbiotic supplement (NCSS), non-challenged, Clinacox medicated (NCCM), challenged, non-medicated (CNM), challenged, synbiotic supplement (CSS), and challenged, Clinacox medicated (CCM) with 6 replicates per group and 30 poults per replicate. Treatment groups were placed in 2x3 factorial arrangement at The Ohio State University's OARDC turkey facility. On day of hatch (dOH), the CSS, CCM, and CNM treatment groups were challenged with *Salmonella enterica* Enteritidis ($4x10^4$ CFU/poult). On d16, the CNM, CSS, and CCM treatment groups were challenged with a mix of *Eimeria adenoeides* and *Eimeria meleagrimitis*. On d21 and d28, five poults from pen (n=30 poults per group) were euthanized and lesion scored for *Eimeria.* Additionally contents were collected from the duodenum, jejunum, ileum, and ceca for microbial analysis by qPCR. The remaining poults were grown out to 45 days of age.

DNA extraction was performed on the jejunum, ileum, and ceca digesta samples taken on d21 and d28. The samples were weighed, in equal volumes, and added to a "Master Mix" tube. The digesta samples were mixed together. Then 0.3g of the mixed digesta was added to a screwcap 2.0mL microcentrifuge tube with 0.2g of zirconia beads (0.1mm). Following the protocol from (Arthur et al. 2012) with several modifications, the DNA was extracted from each sample. Pure culture bacterial samples were also extracted for DNA. DNA quality and quantity was measured using Synergy HTX, Multi-Mode Reader (BioTek). Samples were diluted to 20ng/µL for qPCR and PCR analysis.

For each of the bacterial targets, primer sequences were found in published literature. All primer sequences and reference sources are listed in table 1.

Table 1. Primer sequences for bacterial targets

Target	Target Gene	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')	Reference
Campylobacter jejuni (Species)	*hsp60*	CAA GTT GCT ACA ATC TCA GCC A	GAT AAC ACC ATC TTT GCC CAC T	(Park et al., 2011)
Clostridium perfringens (Species)	*cpa*	TGCATGAGCTTCAATTAGG T	TTAGTTTTGCAACCTGCTGT	(Heikinheimo and Korkeala, 2005)
Salmonella (Genus)	*invA3*	AACGTGTTTCCGTGCGTAA T	TCCATCAAATTAGCGGAGG C	(Cheng et al., 2008)

E. coli (APEC) (Genus)	*sitA*	ATCGGCATTACGTTGGTAG G	TCTCAATGGGGTTCCAGAA G	(Mellata et al., 2009)
Lactobacillus (Genus)	16S rRNA	CAC AAT GGA CGM AAG TCT GAT G	CGC CAC TGG TGT TCT TCC AT	(Songjinda et al., 2007)
Bifidobacteriu m (Genus)	16S rRNA	CGC GTC CGG TGT GAA AG	CCC CAC ATC CAG CAT CCA	(Delroisse et al., 2008)
Lactobacillales (Order)	16S rRNA	CTTGAGTGCAGAAGAGG	CACTGGTGTTCTTCCAT	(Videnska et al., 2013)
Firmicutes (Phylum)	16S rRNA	AAACTCAAAGGAATTGACG G	ACCATGCACCACCTGTC	(Bacchetti De Gregoris et al., 2011)
Bacteria (Domain)	16S rRNA	CGGTCCAGACTCCTACGGG	TTACCGCGGCTGCTGGCAC	(Lee et al., 1996)

All qPCR assays were optimized using QuantiTect SYBR Green PCR Kit (Qiagen) and CFX Connect Real Time System (BioRad). Each run consisted of Each run consisted of a 25µL volume reaction, composed of 12.5µL of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), forward and reverse primers (Table 2), 40ng of sample DNA and water to volume. Each run was optimized to the following conditions, 95℃ for 15 minutes followed by 40 cycles of denaturation and annealing specific to each target. Plate reads were conducted at the end of each annealing cycle. After the 40 cycles were completed, samples were subjected to melt curve analysis which ran from 60 or 65ºC to 95℃, increasing in 1.0℃ increments and holding for one minute at each degree.

Table 2. Bacterial target qPCR conditions

Target	Fragment Size	Primer Concentrations	Denaturation Time	Annealing Conditions
Salmonella	262bp	400nM F & R	20 seconds	55°C for 30 seconds
Lactobacillus	358bp	450nM F & R	25 seconds	61°C for 30 seconds
Bifidobacterium	244bp	400nM F & R	15 seconds	60-61°C for 20-25 seconds
Lactobacillales	74bp	100nM F & 160nM R	10 seconds	52°C for 15 seconds
Firmicutes	114bp	90nM F & 140nM R	15 seconds	53°C for 15 seconds
Bacteria	~200bp	75nM F & R	15 seconds	60°C for 20 seconds

For analysis, the threshold was raised to 100 RFU with each reaction before using the C_q values for calculating the relative abundance of each target. After all targets had been completed, the qPCR runs were reviewed and any samples that were fell below the 100 RFU line were assigned a C_q value of 40 if they had RFU values above 40, to help reduce bias in the ratio fold change calculations. If their C_q values were not above 40, the samples were not included in the analysis for the target that they had no amplification occur. Each run had a standard dilution series along with non-template controls (NTC) included. The standard dilution series was created from either a NCNM sample or pure culture and was used to generate the slope (y=mx+b) of the line. From each slope, the efficiency percentage for the run was calculated along with the coefficient of determination (R^2). Ideal values range from 90-105% for E% and >0.98 for R^2. All analysis was conducted using CFX Manager (BioRad). Random samples were taken from each plate and run on a TBE gel with a NTC and standard sample to confirm the melt curve analysis results and reaction specificity. Using the C_q values from each qPCR, the data was analyzed using the Pfaffl method, shown in equation 1.

All data was normalized using the 16S rRNA *Bacteria* domain as the housekeeping gene. The values derived from the Pfaffl method calculate the ratio fold changes of bacterial

abundance in the treated samples relative to those of the NCNM group. For the final analysis, all of the ratio fold changes were transformed into Log_2 values to differentiate between the increases or decreases for the target of interest. Before transforming the data, high outliers were identified and removed for each target by group. After the transformation, low outliers were identified as over 3 STD from the population (all groups by target) mean and were removed before statistical analysis was conducted.

For *E. coli* (APEC), *C. perfringens* (CP) and *Campylobacter jejuni* (CJ) detection, samples were subjected to PCR rather than qPCR due to the small number of samples likely to test positive for either target, based on the results determined by the *Salmonella* qPCR.

For APEC detection, each reaction contained 1X of Standard *Taq* Reaction Buffer, 2mM of $MgCl_2$, 200µM of dNTP's, 400nM of each primer, 1.25 units of *Taq* DNA Polymerase, 100ng of DNA and water to volume of 25µL. The PCR conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 20 seconds, 58°C for 20 seconds and 72°C for 20 seconds followed by an additional 10 minutes at 72°C for extension. Samples were subjected to electrophoresis using a 2.0% TBE gel for a time of 50 minutes at 70 volts. Positive samples were identified by the presence of a DNA band at the size of 196bp. Each gel had a positive control sample and 100bp DNA ladder for identifying positive samples, and these control measures were used on all gels.

For CP detection, each reaction contained 1X of Standard *Taq* Reaction Buffer, 2mM of $MgCl_2$, 160µM of dNTP's, 500nM of each primer, 2.0 units of *Taq* DNA Polymerase, 100ng of DNA and water to volume of 25µL. The PCR conditions were 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 51°C for 30 seconds and 72°C for 30 seconds followed by an additional 10 minutes at 72°C for extension. Samples were subjected to electrophoresis using a 1.0% TBE gel along with the same conditions as APEC. Positive samples were identified by the presence of a DNA band at the size of 382bp.

For CJ detection, each reaction contained 1X of Standard *Taq* Reaction Buffer, 2mM of $MgCl_2$, 160µM of dNTP's, 350nM of each primer, 2.0 units of *Taq* DNA Polymerase, 100ng of DNA and water to volume of 25µL. The PCR conditions were 94°C for 2 minutes, followed by 35 cycles of 94°C for 10 seconds, 57ºC for 10 seconds and 72°C for 10 seconds followed by an additional 5 minutes at 72°C for extension. Samples were subjected to electrophoresis using a 2.0% TBE gel for a time of 30 minutes at 100 volt. Positive samples were identified by the presence of a DNA band at the size of 90bp.

All qPCR data was subjected to an Oneway Analysis of Variance (ANOVA) using the JMP Pro13 Software (JMP Software, SAS Inc., 2018). Ratio fold changes, expressed as log_2 values, between the groups were compared using Student's *t*-test ($p \leq 0.05$) with significant differences distinguished by different subscripts in the graphs. For the results the group averages are expressed as log_2 fold change ± standard error. Using JMP, box plots were generated for each target. For the presence or absence of the bacterial targets detected by PCR, the number of positive samples were compared between groups using the Chi-Square test ($p \leq 0.05$).

RESULTS AND DISCUSSION:

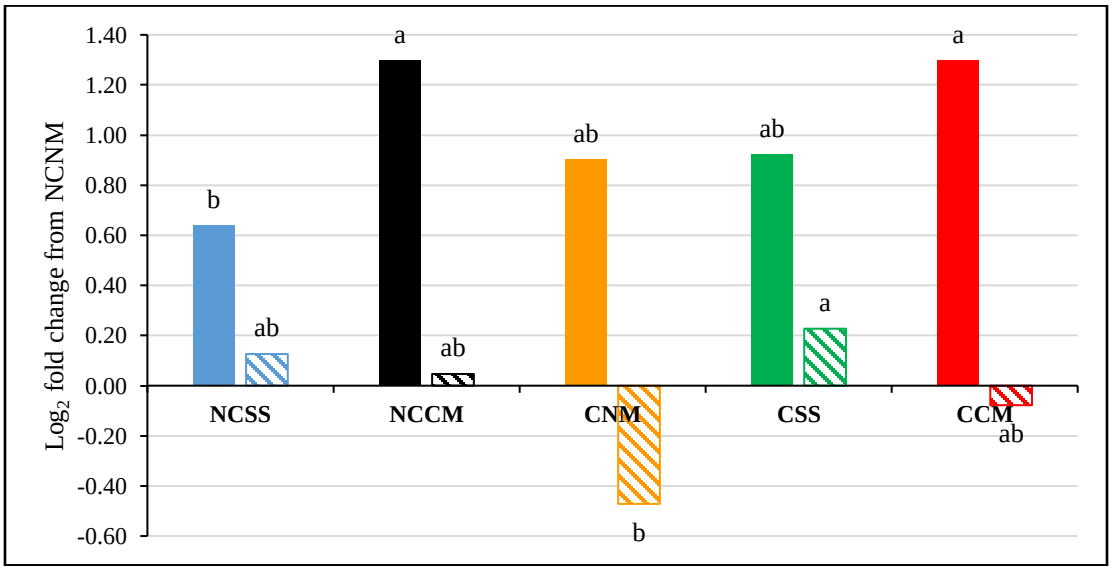
Table 3: qPCR Performance Parameters for d21 and d28 Analysis

Target	d21 E%	d21 R^2 Value	d28 E%	d28 R^2 Value
Salmonella	62.5	0.9840	105.0	0.9920
Lactobacillus	93.4	0.9980	91.1	0.9923
Bifidobacterium	111.6	0.9931	103.5	0.9867
Lactobacillales	83.0	0.9999	97.1	0.9962

Firmicutes	90.7	0.9998	95.8	0.9935
Bacteria	99.2	0.9960	102.4	0.9965

There was a degree of variation between the performance parameters when running the d21 and d28 samples, as highlighted in Table 3. Despite the low E% for d21 *Lactobacillales*, the d28 E% was ideal, and this type of discrepancy between plates was frequently encountered when conducting qPCR. When running *Bifidobacterium*, with the d21 samples the annealing temperature was set to 60ºC and the test run returned a standard curve with an E% of 105.5% with an R^2 value of 0.9870. After running the d21 samples, and seeing high E percentages, the annealing temperature was raised to 61ºC and the annealing time extended from 20 to 25 seconds for the d28 samples. The test run returned a standard curve with an E% of 103.5% and an R^2 value of 0.9867. The same standard dilution set was used for both of the d28 qPCR analysis plates, but the average E% was 128.0%, with an R^2 value of 0.9970 between the plates. Looking at the melt curve analysis, there were no problems with the primer performance and the C_q values did not vary significantly between runs when the same samples were run twice. For the d28 analysis, the curve generated by the test run was used for reporting the performance parameters. When running the *Salmonella* qPCR for both d21 and d28, there were less than 10 samples that were deemed positive by the SYBR Green I fluorescence. SYBR Green I is a non-specific dye that binds to any double stranded fragments, which can include false positive samples or primer dimers. The melt curve analysis and electrophoresis confirmed that all samples that had amplified during the reaction were not actually positive for *Salmonella*. Therefore, no samples from either d21 or d28 were found to have a detectable level of *Salmonella* present.

Figure 1: Fold changes between treatment groups relative to NCNM for Firmicutes on d21 and d28



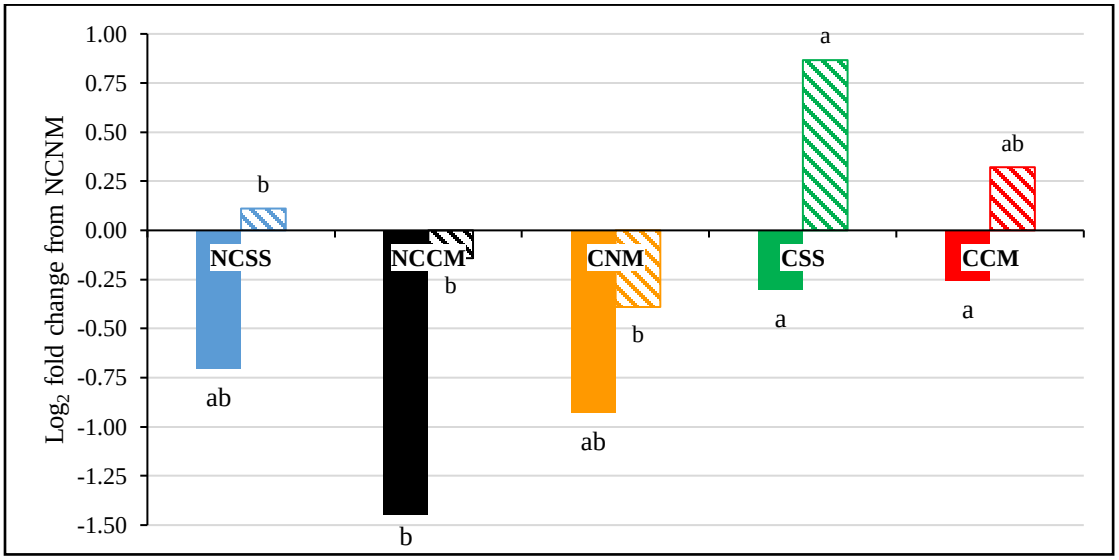
*d21 values are represented by solid bars, whereas d28 values are represented with the striped bars [a,b]bars with different superscripts are significantly different at $p \leq 0.05$

On d21, there was a higher fold change of the Firmicutes population in all treatment groups compared to NCNM. The fold increases were 0.64±0.20, 1.30±0.18, 0.90±0.18,

0.92±0.18 and 1.30±0.19, respectively. There were significant differences ($p \leq 0.05$) between the NCSS and the Clinacox medicated groups. Both of the Clinacox medicated groups had twice the number of Firmicutes compared to the NCNM, whereas the NCSS had the lowest fold change of Firmicutes detected among the treatment groups.

On d28, there was a -0.47±0.25 fold (28%) reduction in the CNM group and a -0.08±0.23 (5%) reduction in the CCM. The NCSS, NCCM and CSS had fold increases of Firmicutes compared to NCNM (0.13±0.21, 0.05±0.20 and 0.23±0.19 respectively). The synbiotic supplement treatment groups had the highest increases, with the CSS having the largest increase for d28. There was a significant difference ($p \leq 0.05$) between the CSS and CNM group.

Figure 2: Fold changes between treatment groups relative to NCNM for *Lactobacillales* on d21 and d28

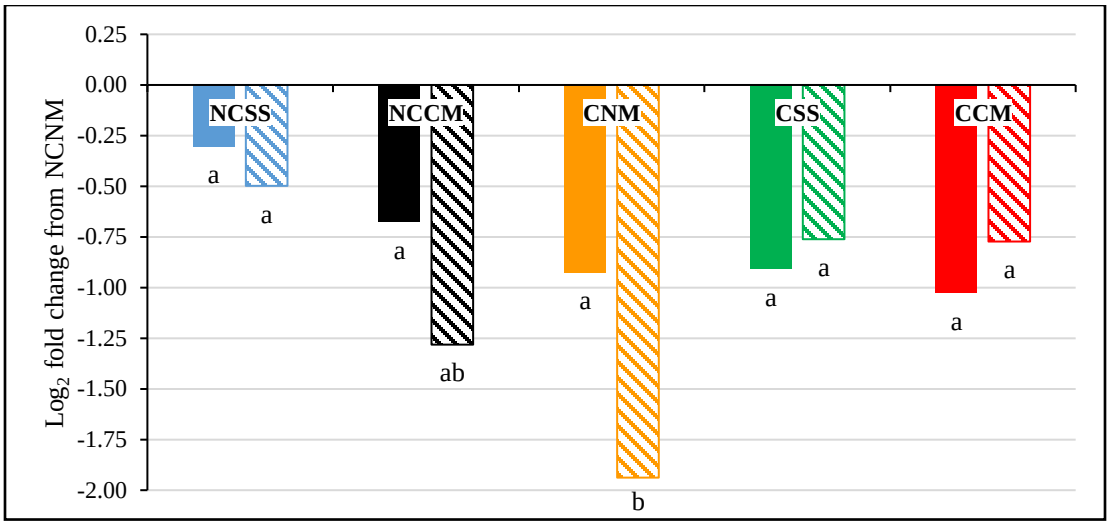


*d21 values are represented by solid bars, whereas d28 values are represented with the striped bars [a,b]bars with different superscripts are significantly different at $p \leq 0.05$

For the *Lactobacillales* (LAB) order there were reductions in all of the groups relative to the NCNM on d21. The reductions seen were -0.71±0.36 (39%), -1.45±0.31 (63%), -0.93±0.32 (47%), -0.30±0.38 (19%) and -0.26±0.30 (16%) respectively. Groups that were challenged with *Eimeria* and treated with the synbiotic supplement or Clinacox had the lowest reductions of LAB, and were significantly different ($p \leq 0.05$) from the NCCM. The NCCM had the highest fold reduction compared to the NCNM.

On d28, the NCSS (0.11±0.33), CSS (0.87±0.25) and CCM (0.32±0.24) had fold increases in LAB, with CSS being significantly higher ($p \leq 0.05$) than NCSS, NCCM and CNM. There were reductions of -0.14±0.25 (9%) and -0.39±0.27 (24%) in the NCCM and CNM, respectively, with the highest reduction seen in the CNM group.

Figure 3: Fold changes between treatment groups relative to NCNM for *Lactobacillus* on d21and d28

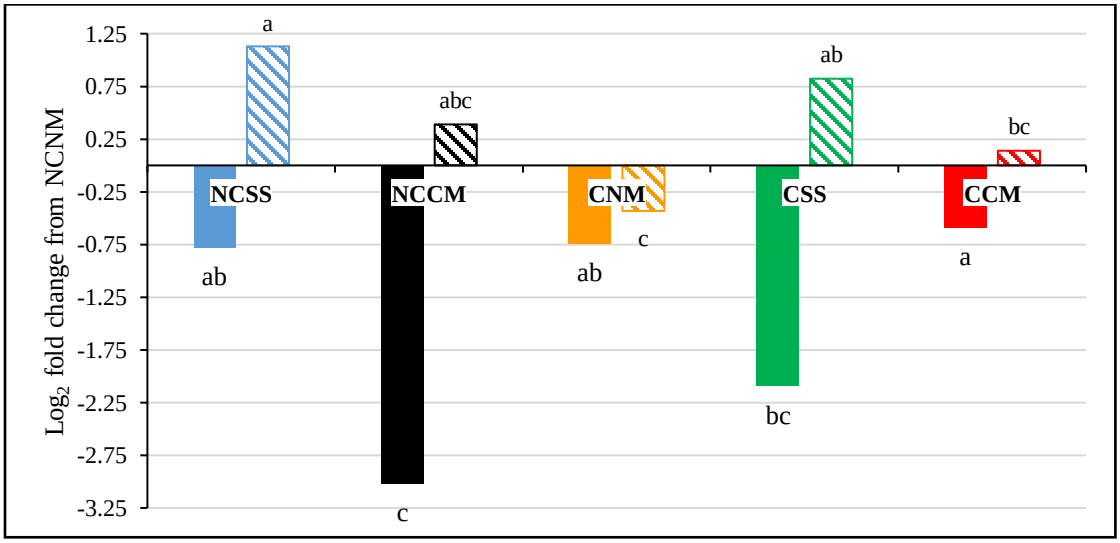


*d21 values are represented by solid bars, whereas d28 values are represented with the striped bars [a,b]bars with different superscripts are significantly different at p≤0.05

With the *Lactobacillus* genus on d21, all of the groups had fold change reductions relative to the NCNM. The reductions were -0.30±0.34 (19%), -0.68±0.31 (37%), -0.92±0.27 (47%), -0.91±0.30 (47%) and -1.03±0.21 (51%) respectively. All reductions were highest in the challenged groups, with the CNM and CSS having smaller reductions than the CCM. The lowest reduction seen across all groups was with the NCSS. There were no significant differences between the groups.

As seen with d21, on d28 all groups continued to have reductions for the *Lactobacillus* genus relative to the NCNM. The reductions were -0.50±0.29 (29%), -1.28±0.35 (59%), -1.94±0.30 (74%), -0.76±0.40 (41%) and -0.77±0.38 (41%) respectively. The lowest reductions were in the NCSS, CSS and CCM groups, all of which were significantly different (p≤0.05) from the CNM. The lowest reduction, seen across all groups was with the NCSS group, as seen with d21.

Figure 4: Fold changes between treatment groups relative to NCNM for *Bifidobacterium* on d21 and d28



*d21 values are represented by solid bars, whereas d28 values are represented with the striped bars [a,b]bars with different superscripts are significantly different at $p \leq 0.05$

On d21, all groups had reductions for the *Bifidobacterium* genus relative to the NCNM. The reductions were -0.79±0.34 (42%), -3.02±0.63 (88%), -0.75±0.49 (40%), -2.10±0.60 (77%) and -0.59±0.53 (34%) respectively. The greatest reductions were in the NCCM and CSS groups with the lowest reduction seen in the CCM. There were significant differences ($p \leq 0.05$) between the NCSS, CNM, CCM and NCCM groups along with the CSS and CCM groups.

On d28, the reductions for the *Bifidobacterium* genus from d21 became fold increases in all groups except the CNM. The values were 1.13±0.24, 0.39±0.39, -0.43±0.35 (26% reduction), 0.82±0.25 and 0.14±0.28 respectively. The highest increases were in the synbiotic supplement groups with the NCSS group having a 1-fold increase compared to the NCNM. There were significant differences ($p \leq 0.05$) between the NCSS, CSS and CNM groups along with the NCSS and CCM groups.

Table 4: Number and percent of samples positive for APEC or CP

Group	d21 APEC	d28 APEC	d21 CP	d28 CP
NCNM	9/29 (31.0%)[b]	12/28 (42.9%)[a]	0/29 (0%)[a]	0/28 (0%)[a]
NCSS	19/29 (65.5%)[a]	3/30 (10.0%)[b]	1/29 (3.4%)[a]	0/30 (0%)[a]
NCCM	7/29 (24.1%)[b]	6/29 (20.7%)[a,b]	3/29 (10.3%)[a]	0/29 (0%)[a]
CNM	7/30 (23.3%)[b]	5/22 (22.7%)[a,b]	1/30 (3.3%)[a]	0/22 (0%)[a]
CSS	0/30 (0%)[c]	13/30 (43.3%)[a]	0/30 (0%)[a]	0/30 (0%)[a]
CCM	5/29 (17.2%)[b]	11/28 (39.3%)[a]	0/29 (0%)[a]	1/28 (3.6%)[a]

For the d21 APEC analysis, there were significant difference between the groups. The NCSS had the highest number of samples that tested positive for the *sitA* gene, whereas CSS had no positive samples. There was no significant differences between the NCNM, NCCM, CNM and CCM groups, but there were reductions seen across all groups with the *Eimeria* challenge compared to the non-challenged groups. For d28, there continued to be a significant difference between the NCSS and CSS groups. The NCSS group had the least number of positive samples, while the NCNM, CSS and CCM groups had significantly higher numbers of positive samples. A point of consideration is the primers used for this target. Iron is an essential cofactor that is needed for most bacteria to survive, and *sitABCD* is one of the three iron acquisition systems found in *E. coli*. Given the importance of this system, it is possible that *sitA* could be found in not only APEC species but also nonpathogenic *E. coli* species (Schouler et al., 2012).
The number of samples that tested positive for CP on either d21 or d28 was minimal and had no significant differences between the groups. The highest number of positive samples was seen on d21 with NCCM. There was no positive samples detected either day for NCNM or CSS.

No samples on either d21 or d28 had *Campylobacter jejuni* detected.

CONCLUSIONS:

Several conclusions can be inferred from these results. Analyzing the Firmicutes Phylum analysis, all groups had $\log_2$ fold increases compared to the NCNM group. Additionally, the Clinacox medicated groups had 1-fold higher increases at d21 compared to the NCNM group, which was significant. By d28, the $\log_2$ fold changes were lowest for the Clinacox medicated groups compared to the rest of the treatments. Interestingly, the CNM group had the largest negative $\log_2$ fold change of Firmicutes. Compared to d21, NCSS and CSS on d28 had the lower

log_2 fold changes among treatment groups. Firmicutes is one of the most prevalent microorganisms in the GIT of poultry, and is reported to have a positive relationship to energy harvested from the diet in mice and humans and increases in Firmicutes has been linked to better nutrient absorption (Oakley et al. 2014). The treatment of the synbiotic supplement may be beneficial as it is associated with a lower reduced log_2 fold change in d28 compared to d21 of the NCSS and CSS groups.

Analyzing the LAB order analysis, reductions in log_2 fold changes were seen in every treatment group compared to the NCNM group. Significantly different ($p \leq 0.05$) from the NCCM on d21, the CSS and CCM groups had the lowest reduction of LAB. This interaction may be due to the challenge of *Eimeria.* On d28, the CSS group had a significant and largest log_2 fold change compared with the NCNM group.

There were negative reductions among all treatment groups compared to NCNM on d21 and d28 for the *Lactobacillus* genus. All the challenged groups compared to the non-challenged groups had larger reductions. *Lactobacillus* is another predominant component of the GIT. *Lactobacillus* in the GIT of poultry is shown to increase the activity of certain enzymes such as proteases, trypsin, and lipases (Clavijo et al., 2018). Feeds and probiotics may encourage the production of *Lactobacillus* in the GIT. Though in this study, there were no significant differences amongst the groups to verify this thought.

Bifidobacterium genus is thought to have similar effects as the *Lactobacillus* genus (Clavijo et al., 2018). On d21, all treatments had reductions compared to the NCNM group. On d28, the reductions amongst all treatment groups become positive fold changes except in the CNM group. This group did not receive any synbiotic supplement or Clinacox treatment. Therefore, on d21 groups all groups that received the synbiotic and Clinacox treatments had log_2 fold increases. This may conclude that there was a benefit to birds treated with the synbiotic supplement due to the increase in *Bifidobacterium*, and similarity to birds treated with Clinacox.

The challenged treatment groups were inoculated with *Salmonella.* Therefore, it would be expected for some of the samples from those treatment groups to test positive for *Salmonella*. As stated in the results, none of the samples from either d21 or d28 were found to have detectable levels of *Salmonella.* This may be due to the dose of *Salmonella* given to the poults was not high enough to cause an infection. A study concerning an acceptable dose to induce an infection would be warranted.

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